FULL PAPER *Parasitology*

The Genetic Diversity of Merozoite Surface Antigen 1 (MSA-1) among *Babesia bovis* Detected from Cattle Populations in Thailand, Brazil and Ghana

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ABSTRACT. In the present study, we screened blood DNA samples obtained from cattle bred in Brazil (n=164) and Ghana (n=80) for *Babesia bovis* using a diagnostic PCR assay and found prevalences of 14.6% and 46.3%, respectively. Subsequently, the genetic diversity of *B. bovis* in Thailand, Brazil and Ghana was analyzed, based on the DNA sequence of merozoite surface antigen-1 (MSA-1). In Thailand, *MSA-1* sequences were relatively conserved and found in a single clade of the phylogram, while Brazilian *MSA-1* sequences showed high genetic diversity and were dispersed across three different clades. In contrast, the sequences from Ghanaian samples were detected in two different clades, one of which contained only a single Ghanaian sequence. The identities among the *MSA-1* sequences from Thailand, Brazil and Ghana were 99.0–100%, 57.5–99.4% and 60.3–100%, respectively, while the similarities among the deduced MSA-1 amino acid sequences within the respective countries were 98.4–100%, 59.4–99.7% and 58.7–100%, respectively. These observations suggested that the genetic diversity of *B. bovis* based on *MSA-1* sequences was higher in Brazil and Ghana than in Thailand. The current data highlight the importance of conducting extensive studies on the genetic diversity of *B. bovis* before designing immune control strategies in each surveyed country.
KEY WORDS: *Babesia bovis*, Brazil, Ghana, MSA-1, Thailand.

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Bovine babesiosis is a tick-borne parasitic disease that has a wide global distribution [16]. The disease is caused by different species of bovine *Babesia* parasites, of which *B. bovis* and *B. bigemina* are the 2 major species of economic and clinical significance. The disease caused by these two pathogens requires chemotherapy and is fatal if left untreated [6]. *B. bovis* is thought to be more virulent in cattle than *B. bigemina*, because the former can induce respiratory distress syndrome and neurological disorders in addition to hemolytic anemia [16]. Differences in the clinical presentation are a result of the sequestration of *B. bovis*-infected erythrocytes in capillary beds of vital organs [10]. The currently available methods to control babesiosis include tick control, chemotherapy and live attenuated vaccines [6, 9].

Recently, several groups have focused on the development of vaccines against *B. bovis* based on recombinant antigens [3, 11, 12, 22]. The merozoite surface antigens (MSAs) were considered to be good vaccine candidate, as the antisera raised against the recombinant forms of these antigens blocked the erythrocyte invasion of *B. bovis* [17, 21]. However, the extensive polymorphism shown by MSAs, which might result in altered immune responses, is one of the obstacles for the development of MSA-based sub-unit vaccines [8]. In the previous investigations, it was observed that the MSAs derived from vaccine strains and outbreak isolates (breakthrough isolates) differed in their genetic makeup [4, 14]. In addition, a complete lack of antigenic cross-reactivity was also observed when the MSAs from vaccine strains or breakthrough isolates were analyzed in immunoblot assays using sera obtained from cattle infected with breakthrough isolates or vaccine strains, respectively [14]. Importantly, in the phylogenetic trees, MSA gene sequences of vaccine strains and breakthrough isolates were located in different clades [4, 14]. Therefore, *MSAs* have been considered as novel markers, by which *B. bovis* populations might be divided into antigenically different genotypes [2].

The presence of *B. bovis* among cattle populations in Thailand, Brazil and Ghana has been previously reported [1, 7, 18]. A recent study based on AMA-1, ACS-1, RAP-1, TRAP, pO and MSA-2c genes found that the Brazilian *B. bovis* isolates were genetically conserved, and that all the sequences shared high identities with the respective gene sequences of the Texas strain (T2Bo) of *B. bovis* [18]. However, because these genes are highly conserved among field isolates, the genetic diversity based on these sequences may be uninformative. Among the MSAs, the *MSA-2c* sequences were also relatively conserved [2]. The genetic diversity of *B. bovis* isolates in Thailand was previously analyzed using *rRNA* [7]. However, the *rRNA*-based analyses do not reflect the biological or antigenic differences among field isolates. Although the parasite was reported in Ghana, the genetic variation of the parasite has never been studied in that

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Table 1. PCR amplification of *MSA-1* from *B*. *bovis*-positive DNA samples from Thailand, Brazil and Ghana using three different primer sets

Primer set	Primer sequences (5′ − 3′)		No. of obtained sequences (Accession No.)		
	Forward	Reverse	Thailand	Brazil	Ghana
1[a)]	ATGGCTACGTTT-GCTCTTTTCATTTCAGC	TTAAAATGCAGAGAGAACGAAGTAG-CAGAG	5 (AB763993 − AB763997)	3 (AB763998 − AB764000)	0
2	CAGCCTTGTGCTGTGTTTTGGC	CAGCACCTTGAGTCTGAGGTGATTG	5[b)]	0	1 (AB764011)
3	TGGCAATTACATCGGCGGGTG	GTAGCCACAGTCAATCCGCC	5[b)]	10 (AB764001 − AB764010)	6 (AB764012 − AB764017)

a): Primer set 1 was described by Altangerel *et al*. [2]. b): The sequences obtained by primer sets 2 and 3 in Thailand were identical to those amplified by primer set 1.

country. Therefore, in the present study, we analyzed and compared the genetic diversity of the MSA-1 gene among *B*. *bovis* isolates detected in cattle populations in Thailand, Brazil and Ghana.

MATERIALS AND METHODS

DNA samples: Blood samples were collected from cattle populations of Bahia state in Brazil (n=164) and Accra city in Ghana (n=80) in March 2009 and July 2008, respectively [23]. All animals were clinically normal during sampling. From each sample, 200 μl of whole blood was subjected to DNA extraction using a commercial DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In addition, 24 bovine blood-DNA samples, which were prepared in Thailand and positive for *B*. *bovis* [7], were also used in the present study.

PCR screening for B. bovis: All DNA samples from Brazil and Ghana were analyzed using a previously described *B*. *bovis*-specific nested PCR assay that amplifies spherical body protein-2 (SBP-2) genes [2]. The composition of the PCR mixture and cycling conditions were essentially as described by Altangerel *et al.* [2]. Detection of a 584-bp PCR product by gel electrophoresis was considered positive. PCR amplicons from each country were cloned into a PCR 2.1-TOPO plasmid vector (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions, and for each sample, a single clone was sequenced as previously described [20].

Amplification and sequencing of MSA-1: To amplify the MSA-1 gene, multiple alignments of all available *MSA-1* sequences in GenBank were carried out, after which 3 different primer sets were designed (Table 1). Subsequently, all the *B*. *bovis*-positive DNA samples from Thailand, Brazil and Ghana were subjected to PCR assays for the amplification of *MSA-1*. *B*. *bovis*-positive DNA samples (2 μl) were added to 28 μl of reaction mixture containing 200 μM of each dNTP (Applied Biosystems, Branchburg, NJ, U.S.A.), 1 × PCR buffer (Applied Biosystems), 10 μM of forward and reverse primers, 1.25 units of *Taq* polymerase (Applied Biosystems) and double distilled water. Following an enzyme activation step at 95°C for 5 min, the PCR mixture was subjected to 45 cycles of 95°C for 30 sec, 52°C for 1 min and 72°C for 2 min, followed by a final elongation at 72°C for 10 min. Amplification was confirmed by gel electrophoresis. PCR amplicons were gel-extracted, ligated into a PCR-2.1 TOPO plasmid vector and then sequenced as described earlier [20].

Analysis of MSA-1 gene sequences: MSA-1 gene sequences generated in the present study were analyzed by GENETYX v. 7.0 software (GENETYX, Tokyo, Japan) and the basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/). Sequence identities or similarities between nucleotide or amino acid sequences were calculated using an EMBOSS needle program, which is available online (http://emboss.bioinformatics.nl/cgi-bin/emboss/needle).

Phylogenetic analysis: *MSA-1* sequences determined in the present study, together with the sequences reported from other countries, were used for phylogenetic analysis. The MAFFT online program, which is based on a neighbor joining method, was used to construct the phylogenetic tree [13].

RESULTS

B. *bovis* was detected in cattle from both Brazil and Ghana in the present study. Among the DNA samples, 37 and 24 from Ghana and Brazil, respectively, were positive for *B*. *bovis*, indicating that the infection rate was higher in Ghana (46.3%) than Brazil (14.6%). The *SBP-2* sequences (AB772319–AB772322) determined in the present study showed 87.0–90.9% identity to each other and 87.2–92.7% identity to a T2Bo sequence (XM_001611732).

To clone the MSA-1 gene, all the *B*. *bovis*-positive DNA samples from Thailand, Brazil and Ghana were subjected to PCR amplification using primer set 1 to obtain a full length MSA-1 gene sequence (Table 1). Although 5 and 3 sequences were obtained from Thai and Brazilian samples, respectively, none of the samples from Ghana were positive using this primer set (Table 1). Next, primer set-2 was used to amplify a partial MSA-1 gene fragment. In this case, only a single *MSA-1* sequence was generated from a Ghanaian sample. Subsequently, a third primer pair, primer set 3, was designed, which amplified ten and six *MSA-1* sequences from Brazilian and Ghanaian samples, respectively (Table 1). Although primer sets 2 and 3 also amplified *MSA-1* from the same 5 samples that were positive by primer set 1 from Thailand, the determined sequences were identical to those amplified by primer set 1. Thai sequences that were identified by primer set 1 were 960 bp long, and all sequences amplified using primer set 3 were equal in size (886 bp). In contrast, size differences were observed between the Brazil-

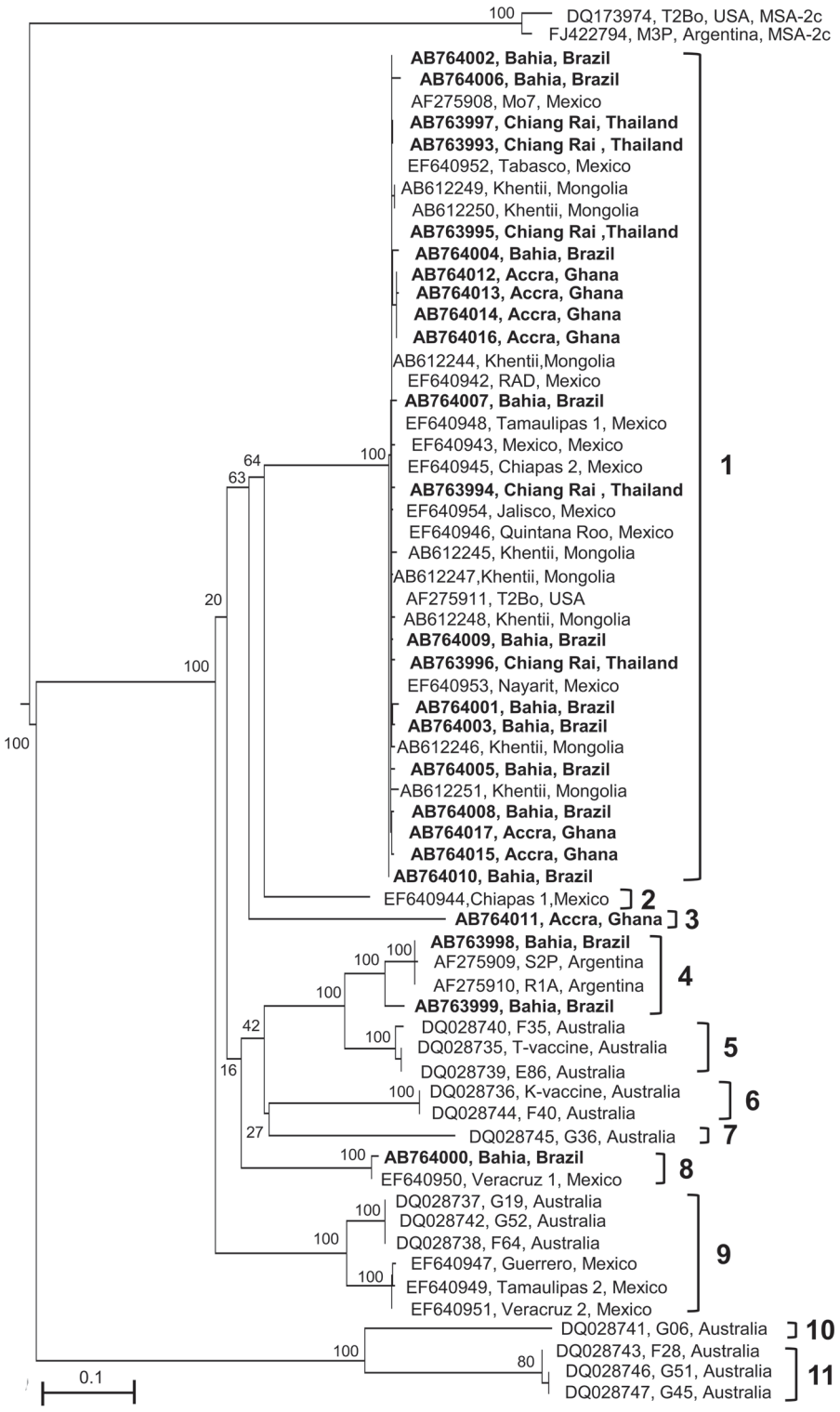

Fig. 1. Phylogenetic analysis of MSA-1 gene sequences. The MSA-1 gene sequences determined in the present study (n=25) and the gene sequences that were already available in GenBank (n=38) were used to construct the phylogram. Two MSA-2c gene sequences were used as an out group. The gene sequences determined in the present study are shown by bold. Bootstrap values are provided at the beginning of each branch. Note that the *MSA-1* sequences determined from *B. bovis* detected in Brazil and Ghana are located in more than one clade.

Group		1						2	3	4		5	6	7	8		9		10	11
Country		Mexico	USA	Mongolia	Brazil	Thai	Ghana	Mexico	Ghana	Argentina	Brazil	Australia	Australia	Australia	Brazil	Mexico	Australia	Mexico	Australia	Australia
	Acc. Num	EF640946	AF275911	AB612245	AB764004	AB763994	AB764012	EF640944	AB764011	AF275910	AB763998	DQ28740	DQ028736	DQ028745	AB764000	EF640950	DQ028737	EF640947	DQ028741	DQ28743
Thailand	AB763993	**100**	**100**	99.3	99.7	99.0	99.3	68.0	58.6	59.4	59.4	60.7	62.7	62.3	61.4	62.9	62.8	63.2	38.9	39.2
	AB763994	**99.0**	**99.0**	98.3	98.6	100	98.3	67.0	58.2	58.7	58.7	60.1	62.7	62.3	60.7	62.3	62.5	62.5	38.9	38.6
	AB763995	**99.7**	**99.7**	99.0	99.3	98.6	99.0	67.6	58.2	59.0	59.0	60.4	62.4	62.0	61.1	62.6	62.5	62.9	41.0	39.2
	AB763996	**99.3**	**99.3**	98.6	99.0	98.3	98.6	67.3	57.9	58.7	58.7	60.1	62.4	61.6	61.1	62.6	62.2	62.5	38.5	38.9
	AB763997	**99.7**	**99.7**	99.0	99.3	98.6	99.0	67.6	58.2	59.0	59.0	60.4	62.4	62.0	61.1	62.6	62.5	62.9	41.0	39.2
Brazil	AB764001	**99.0**	**99.0**	98.6	98.6	97.9	98.3	67.1	57.9	58.7	58.7	60.1	61.7	61.3	60.4	61.9	61.8	62.2	37.7	39.2
	AB764002	**99.7**	**99.7**	99.0	99.3	98.6	99.0	68.0	58.6	59.7	59.7	61.1	62.4	62.0	61.7	62.6	62.5	62.9	39.1	39.8
	AB764003	**100**	**100**	99.3	99.7	99.0	99.3	68.0	58.6	59.4	59.4	60.7	62.7	62.3	61.4	62.9	62.8	63.2	38.9	39.2
	AB764004	**99.7**	**99.7**	99.0	100	98.6	**99.7**	67.6	58.2	59.0	59.0	60.4	62.4	62.0	61.1	62.6	62.5	62.9	38.9	37.7
	AB764005	**99.3**	**99.3**	98.6	99.0	98.3	98.6	68.3	57.7	59.0	59.0	59.7	62.0	61.6	61.1	62.6	62.2	62.5	39.1	38.6
	AB764006	**98.3**	**98.3**	97.6	97.9	97.2	97.6	66.3	57.6	58.7	58.7	60.1	61.7	61.6	61.1	62.6	61.2	61.6	38.5	38.1
	AB764007	**99.3**	**99.3**	98.6	99.0	98.3	98.6	67.3	58.2	58.7	58.7	60.1	62.0	61.6	60.7	62.3	62.2	62.5	38.9	38.6
	AB764008	**99.7**	**99.7**	99.0	99.3	98.6	99.0	68.3	58.6	59.4	59.4	60.7	62.7	62.6	61.4	62.9	63.2	63.5	40.0	38.1
	AB764009	**99.7**	**99.7**	99.0	99.3	98.6	99.0	68.0	58.2	58.8	58.8	60.4	62.4	61.9	61.7	62.9	61.1	62.9	38.5	39.2
	AB764010	**99.3**	**99.3**	98.6	99.0	98.3	98.6	67.6	58.2	58.7	58.7	60.1	62.4	62.0	61.1	62.6	62.5	62.9	38.5	39.2
	AB763998	59.4	59.4	59.0	59.0	58.7	58.7	61.6	53.2	**100**	100	77.7	65.6	65.2	61.4	61.6	55.1	57.5	37.9	38.7
	AB763999	59.7	59.7	59.4	59.4	59.0	59.0	62.3	52.1	**93.9**	94.2	76.4	66.9	65.2	62.7	62.8	56.7	57.7	39.5	36.5
	AB764000	61.4	61.4	61.4	61.1	60.7	60.7	63.3	49.8	61.4	61.4	61.2	59.5	59.9	100	**98.3**	60.5	62.7	42.0	40.3
Ghana	AB764012	99.3	99.3	98.6	**99.7**	98.3	100	67.3	57.9	58.7	58.7	60.1	62.0	61.6	60.7	62.3	62.2	62.5	38.5	36.8
	AB764013	**99.0**	**99.0**	98.3	99.3	97.8	99.7	67.3	57.9	58.7	58.7	60.1	62.0	61.6	60.7	62.3	62.2	62.5	38.8	36.8
	AB764014	99.3	99.3	98.6	**99.7**	98.3	100	67.3	57.9	58.7	58.7	60.1	62.0	61.6	60.7	62.3	62.2	62.5	38.5	36.8
	AB764015	**99.3**	**99.3**	98.6	99.0	98.3	98.6	68.3	58.5	58.8	58.8	60.4	62.4	62.6	61.4	62.9	62.8	63.2	39.2	39.5
	AB764016	99.3	99.3	98.6	**99.7**	98.3	100	67.3	57.9	58.7	58.7	60.1	62.0	61.6	60.7	62.3	62.2	62.5	38.5	36.8
	AB764017	**99.3**	**99.3**	98.6	99.0	98.3	98.6	68.0	58.2	59.7	59.7	61.1	62.4	62.3	61.1	62.6	62.8	63.2	39.2	39.4
	AB764011	58.6	58.6	58.2	58.2	58.2	57.9	**60.3**	100	53.2	53.2	55.1	59.2	52.9	49.8	50.2	57.9	57.6	32.1	34.0

Fig. 2. Similarity analysis of MSA-1 deduced amino acid sequences. The deduced amino acid sequences of *MSA-1* obtained from Thailand, Brazil and Ghana were compared with the sequences from other countries to calculate the percent similarities. Maximum similarities observed with the sequences from other countries are shown in bold.

ian sequences (987–1,020 bp) amplified by primer set 1.

The phylogenetic analysis revealed that the *MSA-1* sequences amplified from Thai samples were highly conserved, and formed a single cluster (clade 1) together with the sequences reported from other countries (U.S.A., Mexico, Mongolia, Brazil and Ghana) (Fig. 1). In contrast, the sequences from Brazilian and Ghanaian samples formed 3 and 2 different clades in the phylogram, respectively. While most of the Brazilian sequences were located in clade 1 together with those from Thailand, 2 other sequences (AB763998 and AB763999) were grouped with Argentine sequences (AF275909 and AF275910) to form clade 4. Furthermore, a single Brazilian sequence (AB764000) was detected in clade 8 along with a Mexican sequence (EF640950). On the other hand, the *MSA-1* sequences from Ghanaian samples formed 2 different clades: clade 1, including 6 of the seven Ghana sequences and clade 3, which contained only a single *MSA-1* sequence (AB764011) (Fig. 2).

The percent similarities of the deduced amino acid sequences of MSA-1 showed that all 5 sequences from Thailand were closer (99.0–100% similarity) to those of T2Bo (AF275911) and a Mexican isolate (EF640946) (Fig. 2). All of the Brazilian sequences, which clustered in clade 1, shared higher similarities (98.3–100%) with MSA-1 from a Mexican isolate (EF640946) and T2Bo (AF275911), than with sequences from other countries. However, a single Brazilian sequence (AB764004) in clade 1 was closely related (99.7% similarity) to the Ghanaian sequence (AB764012). While the single Brazilian sequence (AB764000) found in clade 8 was closer to a Mexican sequence (EF640950), showing 98.3% similarity, the sequences detected in clade 4 (AB763998 and AB763999) exhibited higher similarities (100% and 93.9%, respectively) to an Argentine sequence (AF275910). In contrast, the Ghanaian sequence (AB764011) in clade 3 was closer to a Mexican sequence (EF640944), but the similarity was only 60.3%. Among the Ghanaian sequences in clade 1, three (AB764013, AB764015 and AB764017) were closer to that of T2Bo and a Mexican (EF640946) sequence (99.0–99.3% similarities), while the remaining 3 shared high similarity (99.7%) with a Brazilian sequence (AB764004) determined in the present study (Fig. 2).

The *MSA-1* sequences from Thai samples were highly conserved and showed 99.0–100% identity and 98.4–100% similarity at the nucleotide and amino acid levels, respectively (Fig. 3). In contrast, the genetic diversities within Brazilian and Ghanaian sequences were higher than was found in Thai samples, as the *MSA-1* identities ranged from 57.5–99.4% and 60.3–100%, respectively. Additionally, the similarities between the deduced MSA-1 amino acid sequences determined from Brazilian and Ghanaian samples were 59.4–99.7% and 58.7–100%, respectively.

DISCUSSION

The present study describes the PCR detection of *B. bovis* from cattle from selected locations in Brazil and Ghana, which are located on 2 different continents. Subsequently, the genetic diversity among the isolates from Thailand, Brazil and Ghana was analyzed based on the MSA-1 gene sequence. The prevalence of *B. bovis* in the surveyed location in Ghana (46.3%) was higher than that of Brazil (14.6%). A previous study conducted in Ghana and Brazil using nested PCR assays found that 90.0% and 82.7% animals were positive for *B. bovis*, respectively [1]. In contrast, when a real-time PCR assay was used to detect the parasite in Brazil, the prevalence in 2 different sampling areas was 10% and 95.9% [19]. These discrepancies might be due to the differences in the endemicity of *B. bovis* in the sampling locations.

This is the first report on the genetic diversity of *B. bovis* in Ghana and also the first to use MSAs as a genetic marker in samples from Thailand. Therefore, we investigated the variations that might be present in the MSA-1 gene of *B. bovis* populations in these countries. Although many *B. bovis*-positive samples obtained from each country were studied, the MSA-1 gene fragments were amplified only from a proportion of the samples. The previously reported, highly specific, nested PCR assay for *B. bovis* detection would have amplified the target region, even if the concentration of template DNA was very low [2]. In contrast, our single step PCR assays may not amplify the MSA-1 gene from DNA samples with a low concentration of *B. bovis* genomic DNA. In contrast to the genes in MSA-2 locus, in which the signal peptides among *MSA-2c* as well as among *MSA-2a1*/*MSA-2a2*/*MSA-2b* sequences are highly conserved, polymorphism is common among nucleotides in the signal peptide regions of *MSA-1* from different *B. bovis* isolates [14]. Therefore, MSA-1 gene sequences from different isolates of *B. bovis* cannot be amplified by a single primer set. This might also explain the low amplification rate and the differences in the amplification efficiency of each primer set.

The present findings showed that the MSA-1 gene fragments amplified from Thai samples were highly conserved. However, we cannot be conclusive about the genetic diversity of *B. bovis* isolates in Thailand, as numbers of analyzed sequences were relatively small. In addition, our recent study of *B. bovis* in Mongolia showed that although *MSA-1* sequences clustered in clade 1, together with that of T2Bo, *MSA-2b* sequences formed a clade that was distantly located from T2Bo [2]. When Brazilian *B. bovis* isolates were analyzed in a recent study, the authors found that several genes, including MSA-2c, were highly conserved and shared high sequence identity with the genes from T2Bo [18]. In the present study, however, the MSA-1 gene sequences were found to separate into three different clades. In addition, the identities and similarities among the nucleotide and amino acid sequences were only 57.5–99.4% and 59.4–99.7%, respectively. Therefore, the genetic diversity among Brazilian *B. bovis* isolates was higher than observed previously. MSA-1 gene sequences were reported for the first time in Thailand, Brazil and Ghana in the present study. Therefore, we compared the MSA-1 sequences from these countries with those of available from several other countries to analyze the possible geographical relationship among different isolates. The Brazilian MSA-1 sequences that were found in clades 4 and 6 shared maximum similarities with those from Argentina and Mexico, respectively. In addition, all the

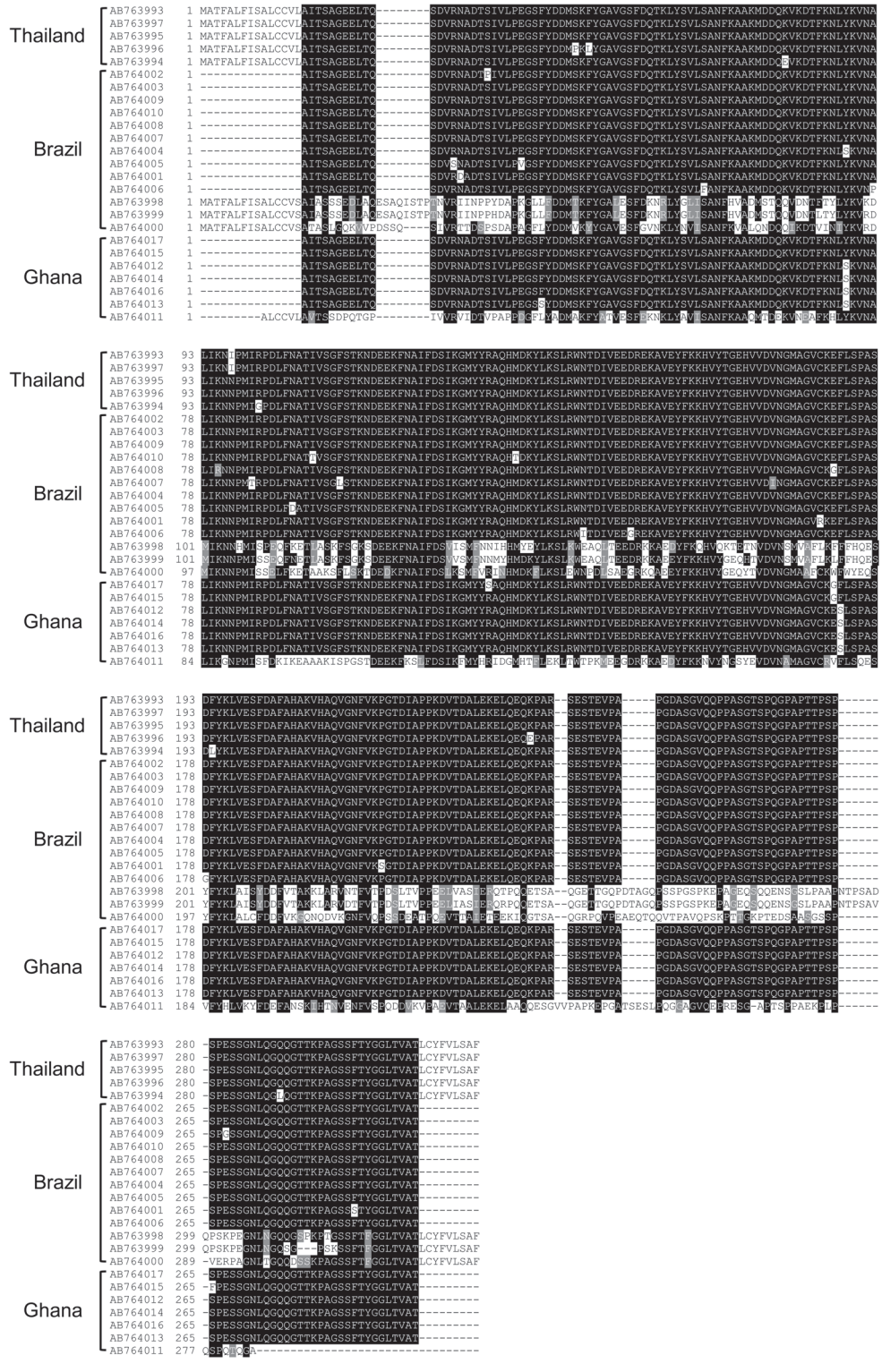

Fig. 3. Multiple alignment of MSA-1 sequences. The deduced MSA-1 amino acid sequences from Thailand, Brazil and Ghana were subjected to multiple alignment. Note that the Thai-MSA-1 sequences were highly conserved, while those of Brazilian and Ghanaian were relatively polymorphic.

Brazilian *MSA-1* sequences in clade 1 were closely related to those from U.S.A. and Mexico, suggesting a possible geographical relationship of *B. bovis* in Central, South and North America.

The Ghanaian sequences were also clustered into 2 different clades, one of which (clade 3) was formed by only a single Ghanaian sequence. Therefore, it is possible that clade 3 might represent *MSA-1* sequences in the African region. However, a large number of *MSA-1* sequences should be determined from several other African countries to confirm our assumption. Similar to the Brazilian sequences, genetic diversity among the *MSA-1* sequences was also very high in Ghana.

Future investigations should be toned to phylogenetically analyze larger number of *MSA-1* sequences and the sequences of other genetic markers, such as *MSA-2b* [2] and *BV80* [15], to provide a clearer picture of the genetic diversity of *B. bovis* in Thailand, Brazil and Ghana.

In the previous studies, the authors concluded that the genetic variation of *MSAs* between the vaccine strains and breakthrough isolates resulted in antigenic differences among them [4, 14]. Consequently, outbreaks of babesiosis are inevitable in vaccinated cattle, if the antigenic variations enable the parasites to evade the immunity induced by the vaccines[5, 9]. Therefore, large-scale investigations to study the genetic diversity of *B. bovis* in Thailand, Brazil and Ghana using several MSAs genes are very important, prior to devising appropriate immune control strategies that may include the selection of suitable vaccine strains or the components of sub-unit vaccines.

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